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Plant Viral Synergism: The Potyviral Genome Encodes a Broad-Range Pathogenicity Enhancer That Transactivates Replication of Heterologous Viruses

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Synergistic viral diseases of higher plants are caused by the interaction of two independent viruses in the same host and are characterized by dramatic increases in symptoms and in accumulation of one of the coinfecting viruses. In potato virus X (PVX)/potyviral synergism, increased pathogenicity and accumulation of PVX are mediated by the expression of potyviral 5′ proximal sequences encoding P1, the helper component proteinase (HC-Pro), and a fraction of P3. Here, we report that the same potyviral sequence (termed P1/HC-Pro) enhances the pathogenicity and accumulation of two other heterologous viruses: cucumber mosaic virus and tobacco mosaic virus. In the case of PVX–potyviral synergism, we show that the expression of the HC-Pro gene product, but not the RNA sequence itself, is sufficient to induce the increase in PVX pathogenicity and that both P1 and P3 coding sequences are dispensable for this aspect of the synergistic interaction. In protoplasts, expression of the potyviral P1/HC-Pro region prolongs the accumulation of PVX (−) strand RNA and transactivates expression of a reporter gene from a PVX subgenomic promoter. Unlike the synergistic enhancement of PVX pathogenicity, which requires only expression of HC-Pro, the enhancement of PVX (−) strand RNA accumulation in protoplasts is significantly greater when the entire P1/HC-Pro sequence is expressed. These results indicate that the potyviral P1/HC-Pro region affects a step in disease development that is common to a broad range of virus infections and suggest a mechanism involving transactivation of viral replication.

INTRODUCTION

Mixed virus infections occur in both plant and animal systems, and doubly infected organisms commonly display increases in disease symptoms and in the accumulation of one or both of the viruses. In animal hosts, mixed virus infections are relatively infrequent and are generally associated with depression of the immune response (Calendar, 1986). In contrast, higher plants are commonly coinfected with multiple viruses, and a number of disease syndromes are caused by the interaction of two independent viruses (Matthews, 1991). Many such synergistic diseases involve a member of the potyvirus group of plant viruses. In potyvirus-associated synergisms, the other virus of the pair may be any of a broad range of unrelated viruses, including pararetroviruses such as cauliflower mosaic virus (Khan and Demski, 1982) and RNA viruses of both the alphavirus supergroup (e.g., potato virus X [PVX]; Rochow and Ross, 1955) and the picornavirus supergroup (e.g., cowpea mosaic virus; Anjos et al., 1992). Several such potyvirus-associated synergistic diseases have been examined in detail, and in each, a dramatic increase in host symptoms has been observed in doubly infected plants compared with singly infected plants. The increase in symptoms is correlated with an increase in the accumulation of the non-potyvirus, but there is no corresponding increase or decrease in the level of the potyvirus (Rochow and Ross, 1955; Calvert and Ghabrial, 1983; Goldberg and Brakke, 1987; Vance, 1991). Such synergistic interactions offer a unique opportunity to investigate the regulation of viral replication and disease because the accumulation and pathogenicity of one virus are altered when it interacts with a relatively simple genetic element, the genome of the coinfecting virus.

The best characterized of the plant viral synergisms is the interaction between PVX and the potyvirus potato virus Y (PVY) in tobacco (Rochow and Ross, 1955; Goodman and Ross, 1974a, 1974b; Vance, 1991). Plants mechanically inoculated with both viruses develop synergistic disease, which is characterized initially by severe vein clearing and then necrosis of the first systemically infected leaf tissue, with a three- to 10-fold increase in the level of PVX compared with singly

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infected plants. A similar synergistic response is induced in coinfections of PVX with several other potyviruses, including the well-studied tobacco vein mottling and tobacco etch viruses (TVMV and TEV, respectively) (Vance et al., 1995).

We have recently shown that PVX–potyviral synergistic disease does not require replication of the potyviral genomic RNA and that the response is mediated by expression of potyviral sequences in the 5′ proximal one-third of the potyviral genome (Vance et al., 1995). This region of the potyviral genome includes the viral genomic 5′ untranslated region (UTR) as well as the coding region for the N-terminal portion of the viral polyprotein, including P1, the helper component proteinase (HC-Pro), and approximately one-quarter of P3 (termed P1/HC-Pro sequence). Here, we report that expression of the TEV P1/HC-Pro sequence also enhances the pathogenicity and accumulation of both tobacco mosaic virus (TMV) and cucumber mosaic virus (CMV), two unrelated plant viruses that cause synergistic disease in conjunction with a potyvirus (Pio-Ribeiro et al., 1978; Clark et al., 1980; Poolpol and Inouye, 1986). We show that in protoplasts, the potyviral sequence prolongs both the accumulation of PVX (−) strand RNA and the expression of a reporter gene from a PVX subgenomic promoter, suggesting that it acts, at least in part, by transactivation of viral RNA replication.

RESULTS

Enhanced Pathogenicity of TMV and CMV in Transgenic Plants Expressing the TEV P1/HC-Pro Sequence

The finding that transgenic plants expressing the P1/HC-Pro region of the potyviral genome develop synergistic disease when infected with PVX (Vance et al., 1995) raised the possibility that many or all potyvirus-associated synergisms might be mediated by this same sequence. To test this hypothesis, we used two other viruses, TMV and CMV, to infect transgenic tobacco line U-6B, which expresses the P1/HC-Pro region of the TEV genome (Carrington et al., 1990). Both viruses can infect tobacco and are known to interact synergistically with a potyvirus. TMV and CMV are both (+) strand RNA viruses in the alphavirus supergroup; however, CMV has a tripartite genome, and many strains also support replication of an associated satellite RNA, whereas the genome of TMV, like that of PVX, is monopartite. Developmentally matched U-6B and vector-only transgenic tobacco plants were inoculated with either TMV or CMV and monitored for symptom development. Both CMV and TMV induced severe symptoms in the U-6B transgenic line, resulting in death of the host plant after several weeks, as shown in Figures 1B and 1D, respectively. In contrast, both viruses induced relatively mild symptoms in the control tobacco plants over the same time period (Figures 1A and 1C, respectively). This result supports the idea that the P1/HC-Pro region of TEV acts as a pathogenicity enhancer for a broad range of viruses.

Enhanced Accumulation of TMV and CMV Genomic RNAs in Transgenic Plants Expressing the TEV P1/HC-Pro Sequence

Accumulation of PVX RNA is enhanced in U-6B transgenic plants (Vance et al., 1995). To determine whether CMV and TMV RNA levels are similarly enhanced in this transgenic line, we compared the levels of viral genomic RNA in systemically infected U-6B plants with those in vector-only transformed plants. In the case of TMV infection, the level of TMV genomic RNA in a severely symptomatic leaf of the U-6B transgenic plant (fourth leaf above the inoculated leaf; Figure 2A, lane 2) was >20-fold higher than that in the corresponding leaf of the control plant (lane 1). Thus, accumulation of TMV genomic RNA, like that of PVX genomic RNA, is enhanced by the expression of the TEV P1/HC-Pro sequence.

In the case of CMV infection, accumulation of several genomic RNAs and a satellite RNA potentially can be affected by expression of the TEV P1/HC-Pro sequence. The CMV genome is divided among three components: RNAs 1 and 2 are monocistronic, whereas RNA 3 is bicistronic, with the internally coded gene expressed from a subgenomic RNA termed RNA 4. Many CMV strains, including the one used here, support the replication of a small satellite RNA. This CMV satellite RNA is completely dependent on the virus for

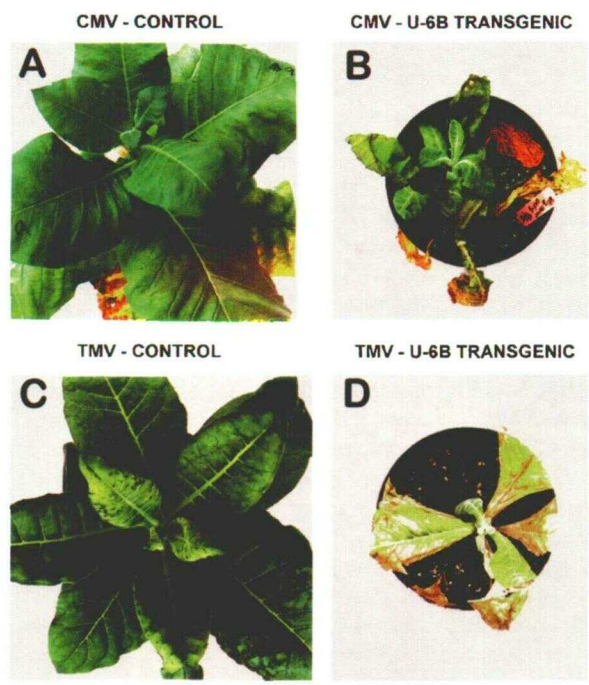

Figure 1. Enhanced Pathogenicity of CMV and TMV in Transgenic Tobacco Expressing the Potyviral P1/HC-Pro Sequence.

(A) and **(C)** Vector-only transformed tobacco plants.
(B) and **(D)** TEV-transformed line U-6B plants expressing the 5′ proximal region of the TEV genome.
Plants were infected with either CMV (**[A]** and **[B]**) or TMV (**[C]** and **[D]**).

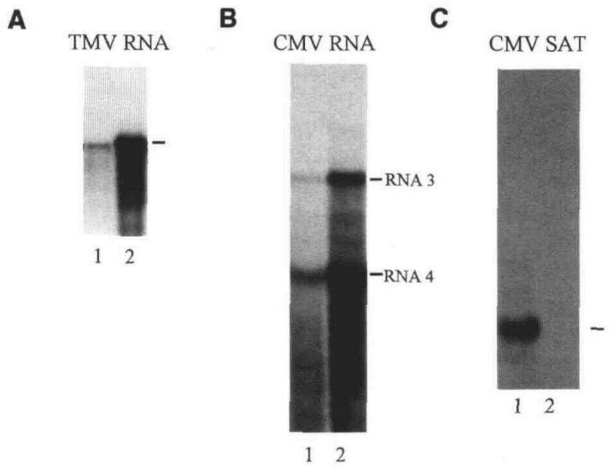

Figure 2. RNA Gel Blot Analysis of CMV and TMV RNAs in TEV-Transformed Line U-6B and Control Plants.

(A) Total plant RNA from TMV-infected transgenic line U-6B (lane 2) or vector-only transformed control plants (lane 1) hybridized with a radioactive probe complementary to TMV genomic RNA. The dash indicates the position of TMV genomic RNA.

(B) Total plant RNA from CMV-infected transgenic line U-6B (lane 2) or vector-only transformed control plants (lane 1) hybridized with a radioactive probe complementary to the 3′ proximal nucleotides common to CMV RNAs 1, 2, 3, and 4. The positions of CMV RNAs 3 and 4 are indicated.

(C) The blot shown in **(B)** was stripped and hybridized with a radioactive probe complementary to the (+) strand of CMV satellite RNA (CMV SAT). The dash indicates the position of CMV SAT.

replication. RNA gel blot analysis showed that levels of CMV RNAs 3 and 4 in a severely symptomatic upper leaf of the TEV-transformed plant (Figure 2B, lane 2) were ~10-fold higher than those in the analogous leaf of the control plant (Figure 2B, lane 1). Longer exposure of the blot showed a similar increase in the levels of RNAs 1 and 2 in the TEV-transformed plant compared with the control (data not shown). These results are similar to those with PVX- and TMV-infected transgenic plants and are consistent with the hypothesis that the potyviral sequence mediates an increase in accumulation of these heterologous viruses. In contrast, the accumulation of CMV satellite RNA was affected in the opposite manner: it was present at high levels in control plants (Figure 2C, lane 1) but was undetectable in the U-6B plants (Figure 2C, lane 2). This result suggests that the TEV sequence affects the balance between CMV genomic and satellite RNA replication, favoring genomic RNA replication.

Expression of the TEV HC-Pro Gene Product Enhances the Pathogenicity of PVX

The P1/HC-Pro region of the TEV genome expressed in U-6B transgenic plants consists of 2670 nucleotides that include the 5′ UTR and the coding region for mature viral proteins P1 and HC-Pro as well as a portion of P3. These proteins are expressed initially as a polyprotein and are then processed by both P1 and HC-Pro autoproteolytic activities (Carrington et al., 1990). To determine whether all or only part of this sequence is required for synergism, we took advantage of the ability of PVX to be used as a vector to express foreign genes. Our strategy was to create PVX–TEV synergism in a system in which PVX itself expresses the P1/HC-Pro sequence and then determine the minimum TEV sequence required for the effect.

We constructed three different PVX vectors by cloning various TEV sequences into our modified version (Sriskanda et al., 1996) of the infectious PVX cDNA clone pTXS (Kavanaugh et al., 1992), and these are shown schematically in Figure

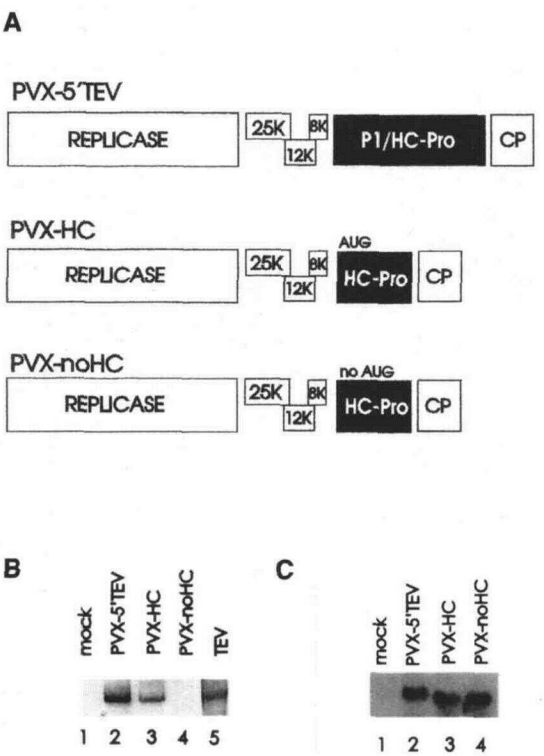

Figure 3. PVX as a Vector to Express TEV Sequences.

(A) Diagram of PVX viral genomic RNAs carrying TEV 5′ proximal sequences. PVX-5′TEV has nucleotides 146 to 2674 of TEV encoding P1, HC-Pro, and part of P3. PVX-HC carries the region encoding HC-Pro plus appropriate translation start and stop codons. PVX-noHC is the same as PVX-HC, except that the initial AUG has been changed to ACG. CP, coat protein.

(B) Protein gel blot showing the level of HC-Pro in mock-infected *N. benthamiana* leaves (lane 1) or in leaves systemically infected with PVX-5′TEV (lane 2), PVX-HC (lane 3), PVX-noHC (lane 4), or TEV (lane 5).

(C) Gel blot of RNA isolated from leaves as given for **(B)** and hybridized with a radioactive probe specific for the TEV HC-Pro sequence.

3A. In these constructs, the expression of the TEV insert is under the control of the authentic PVX coat protein subgenomic promoter, and expression of the coat protein is under the control of an engineered repeated coat protein subgenomic promoter. The vector PVX-5′TEV carries the coding region of the P1/HC-Pro sequence of TEV (nucleotides 146 to 2674). PVX-HC carries only the region encoding HC-Pro (nucleotides 1057 to 2433), with a start codon followed by GCC added at the 5′ end and a stop codon at the 3′ end of the insert so that the mature HC-Pro protein (with two additional N-terminal residues) is made without proteolytic processing. PVX-noHC carries the same TEV sequences as PVX-HC, except that the translation start site was mutated from AUG to ACG.

These three engineered viruses were used to infect *Nicotiana benthamiana* plants. Protein gel blot analysis indicated that HC-Pro accumulated in leaves infected systemically by either PVX-5′TEV or PVX-HC (Figure 3B, lanes 2 and 3, respectively) but as expected was undetectable in leaves infected with PVX-noHC (Figure 3B, lane 4). Although PVX-noHC did not express the HC-Pro gene at the protein level, it had not deleted the sequence from the viral genomic RNA, as shown by gel blot analysis of RNA from systemically infected leaves. A single genomic RNA was detected by using hybridization probes specific either for the TEV HC-Pro sequence (Figure 3C, lane 4) or for the PVX (+) strand genomic RNA (data not shown). Plants infected with PVX-noHC displayed mild symptoms (Figure 4, lower left) compared with the mock-inoculated control plant (Figure 4, upper left). In contrast, infection of plants with either of the viruses expressing the HC-Pro gene product (PVX-5′TEV or PVX-HC) initially caused vein clearing, which was followed by necrosis of systemically infected leaves by 10 days after inoculation and usually killed the plant (Figure 4, upper and lower right, respectively). Together, these results indicate that expression of the HC-Pro gene product, but not the RNA sequence itself, is sufficient to induce the increase in PVX pathogenicity. Furthermore, both P1 and P3 coding sequences are dispensable for the enhanced pathogenicity.

Expression of the TEV P1/HC-Pro Sequence Prolongs the Accumulation of PVX (−) Strand RNA in Protoplasts

In whole plants, PVX–potyviral synergism is characterized by an approximately threefold increase in the accumulation of PVX coat protein and (+) strand RNA and ~10-fold increase in the level of PVX (−) strand RNA (Vance, 1991). This disproportionate increase in accumulation of PVX (−) strand RNA suggests that synergism involves a change in the regulation of viral RNA replication. However, infection of whole plants is asynchronous and involves both replication and movement of the virus. Thus, it is also possible that PVX RNA replication is unaffected and that synergism results solely from a change in the efficiency of viral movement. To determine whether the potyviral sequence affects PVX replication when viral movement is not a factor, we used the PVX vectors described above to examine the effect of TEV P1/HC-Pro expression on the kinetics of accumulation of PVX (+) and (−) strand RNAs in tobacco protoplasts.

Protoplasts were inoculated by electroporation with transcripts of the three engineered PVX cDNAs (Figure 3A, PVX-5′TEV, PVX-HC, and PVX-noHC), and the accumulation of (+) and (−) strand RNAs was assayed by RNA gel blot analysis at different times after inoculation. Because determining kinetics involves comparing RNA levels only within the same inoculated protoplast culture and not between protoplast cultures infected with different PVX vectors, the analysis is independent of infection efficiency. The kinetics of (−) strand RNA accumulation in protoplasts infected with PVX-noHC were similar to those previously reported for the parental PVX strain (Sriskanda et al., 1996), with the level of (−) strand RNA peaking at 24 hr after inoculation and then declining to ~0.2 times the peak level over the next 48 hr, as shown by the RNA gel blot in Figure 5A (bottom row) and graphically in Figure 6. In protoplasts infected with PVX-HC, the level of (−) strand RNA declined only slightly during the same period to a level ~0.8 times the 24-hr peak (Figure 5A, middle row, and Figure 6). The most striking result, however, was obtained in protoplasts infected with PVX-5′TEV. In this case, the level of PVX (−) strand RNA increased over the entire 72-hr period (Figure 5A, top gel); at 72 hr postinocula-

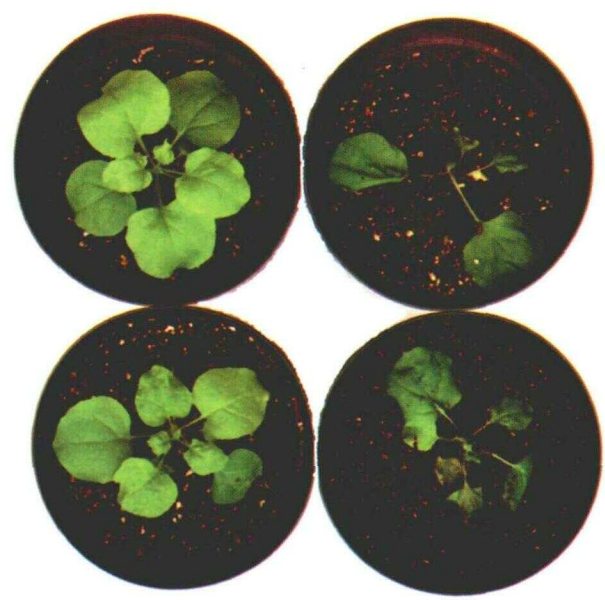

Figure 4. Enhanced Pathogenicity of PVX Expressing the HC-Pro Gene Product.

(Upper left) Mock-inoculated *N. benthamiana* plant.
(Upper right) *N. benthamiana* plant infected with PVX-5′TEV.
(Lower left) *N. benthamiana* plant infected with PVX-noHC.
(Lower right) *N. benthamiana* plant infected with PVX-HC.

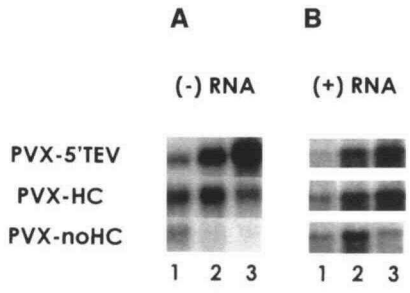

Figure 5. Kinetics of Accumulation of PVX (−) Strand RNA Are Altered by Expression of the TEV P1/HC-Pro Sequence.

RNA gel blot analysis of (+) and (−) strand viral RNAs from tobacco protoplasts infected with PVX-5′TEV (top), PVX-HC (middle), or PVX-noHC (bottom) at 24 (lanes 1), 48 (lanes 2), or 72 (lanes 3) hr after inoculation.

(A) (−) strand viral RNA.

(B) (+) strand viral RNA.

Total RNA was isolated from protoplasts, fractionated by denaturing agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with radioactive probes specific for either the PVX (+) strand RNA **(B)** or (−) strand RNA **(A)**. The regions of the blots containing genomic-length RNAs are shown.

tion, the level of (−) strand RNA was 3.6 times higher than the level at 24 hr (Figure 6).

The accumulation of (+) strand RNA in protoplasts infected with PVX-5′TEV and PVX-HC was also prolonged compared with that in PVX-noHC–infected cells (Figure 5B, compare top and middle rows with the bottom row). However, as is also true in the mixed infections with TEV and PVX and in the transgenic plant system (Vance et al., 1995), the effect of the TEV sequence on (+) strand RNA accumulation was less dramatic than the effect on (−) strand RNA accumulation. Thus, a major effect of TEV P1/HC-Pro expression is to prolong the accumulation of PVX (−) strand RNA. Unlike the synergistic enhancement of PVX pathogenicity, which requires only expression of HC-Pro, the enhancement of PVX (−) strand RNA accumulation is significantly greater when the entire P1/HC-Pro sequence is expressed.

The TEV P1/HC-Pro Sequence Prolongs Expression of a Reporter Gene Cloned into PVX, and This Effect Requires the HC-Pro Gene Product

We previously constructed a PVX reporter virus that provides a very sensitive assay for PVX replication in protoplasts (Sriskanda et al., 1996). In this reporter virus, the luciferase gene replaces the PVX coat protein gene and is expressed from the coat protein subgenomic promoter. Because PVX subgenomic RNA is produced only if RNA replication occurs, the level of luciferase activity reflects the level of PVX genome amplification. When protoplasts are inoculated with this reporter virus, luciferase activity typically increases exponentially during the first 24 hr after inoculation and then levels off (Sriskanda et al., 1996). To test the hypothesis that expression of a reporter gene from a PVX subgenomic promoter would be prolonged in the presence of P1/HC-Pro, we cloned the luciferase gene in place of the coat protein gene on the PVX-5′TEV cDNA and on a mutant version of PVX-5′TEV carrying a mutation (K) in the HC-Pro sequence that eliminates synergism (Shi et al., 1997). Protoplasts were inoculated with transcripts of these two viruses, PVX-5′TEVluc and PVX-5′TEV(K)luc, and luciferase activity was assayed at different times after inoculation. The kinetics of luciferase activity in protoplasts infected with the two reporter viruses were similar for the first 22 hr after inoculation (Figure 7); however, there was a striking difference in luciferase expression levels after that time. Activity from the PVX-5′TEVluc reporter consistently increased ~100-fold between 20 and 72 hr after inoculation, whereas activity from the PVX-5′TEV(K)luc reporter remained approximately constant during the same period (Table 1). These results indicate that gene expression from the PVX coat protein subgenomic promoter is prolonged by expression of the TEV P1/HC-Pro sequence and that HC-Pro is required for this effect.

DISCUSSION

The results presented here indicate that the TEV P1/HC-Pro sequence acts as a general pathogenicity enhancer during

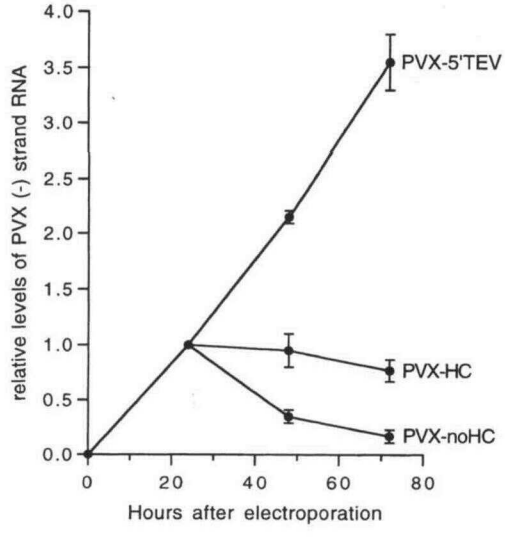

Figure 6. Relative Levels of PVX (−) Strand RNA at Various Times after Infection of Protoplasts with PVX-5′TEV, PVX-HC, or PVX-noHC.

The 48- and 72-hr values are normalized to the 24-hr time point. Each time point is the average of two independent experiments. The error bars represent the standard deviation of the mean.

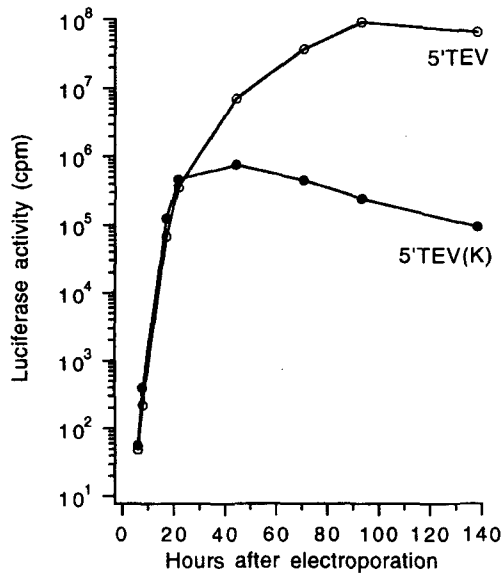

Figure 7. Time Course of Luciferase Activity in Protoplasts Infected with PVX Reporter Viruses.

PVX reporter viruses expressed the TEV P1/HC-Pro sequence (5'TEV) or a mutant version of that sequence (5'TEV(K)) that encodes an altered HC-Pro that fails to support synergism.

infection by viruses from three different groups (PVX, potexvirus; TMV, tobamovirus; CMV, cucumovirus). Because each of these viruses is capable of inducing synergistic disease in mixed infections with a member of the potyvirus group, this result supports the hypothesis that all such potyvirus-associated synergistic diseases, which occur in an evolutionarily diverse range of host plants and involve interactions with many viral groups, are mediated by the same potyviral sequence. The fact that expression of this sequence alters disease induction by each of these unrelated heterologous viruses suggests that it affects a step in viral infection that is common to all of these viruses and thus of general significance.

Our results using PVX as a vector to express TEV sequences indicate that the processed form of HC-Pro is both necessary and sufficient for enhancement of PVX pathogenicity and that it also plays an essential role in the transactivation of PVX replication. However, additional sequences from the P1/HC-Pro region are required for the dramatic effect on the accumulation of PVX (−) strand RNA in protoplasts. We can obtain clues for how HC-Pro and P1 might enhance the pathogenicity and transactivate the replication of heterologous viruses by examining the functions they perform in the TEV infection process.

Both HC-Pro and P1 are multifunctional proteins. P1 has proteinase activity that cleaves the potyviral polyprotein, creating the C terminus of P1 and the N terminus of HC-Pro (Verchot et al., 1991). P1 also functions in *trans* as an accessory factor for genome replication (Verchot and Carrington, 1995) and has RNA binding activity (Brantley and Hunt, 1993). One possibility is that P1 plays a direct role in synergism, perhaps analogous to its role as an accessory factor in TEV replication. Alternatively, it might enhance synergism only indirectly by producing the authentic HC-Pro N terminus by means of its proteinase activity.

HC-Pro has at least three functional domains: an N-terminal domain required for aphid transmission; a central domain involved in pathogenicity, RNA replication, and leaf-to-leaf movement of the virus through the phloem; and a C-terminal domain required for autoproteolytic processing of the HC-Pro C terminus (reviewed in Maia et al., 1996). The central domain of HC-Pro is of particular interest because it is involved in the regulation of both pathogenicity and RNA replication of potyviruses (Atreya et al., 1992; Atreya and Pirone, 1993; Klein et al., 1994; Cronin et al., 1995; Kasschau et al., 1997), and these are the characteristics that are altered in the heterologous virus during synergism. Mutations within the coding region for the central domain of TEV HC-Pro eliminate the ability of the sequence to mediate PVX–potyviral synergistic disease in transgenic plants (Shi et al., 1997). Furthermore, mutations within the central region result in a premature shutoff of TEV RNA amplification, suggesting that HC-Pro may function to prolong potyviral RNA replication (Kasschau et al., 1997). Because expression of P1/HC-Pro prolongs PVX (−) strand RNA accumulation, it is possible that the ba-

Table 1. Luciferase Activity (Counts per Minute) in Protoplasts Infected with PVX Reporter Viruses

	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
Virus	22 Hr	70 Hr	23 Hr	73 Hr	21 Hr	70 Hr	21 Hr	70 Hr
PVX-5'TEVluc	3.6×10^5	3.7×10^7	1.1×10^5	5.0×10^6	5.3×10^3	7.9×10^5	9.2×10^4	8.7×10^6
normalized[a]	1	103	1	45	1	149	1	95
PVX-5'TEV(K)luc	4.6×10^5	4.5×10^5	2.3×10^4	1.9×10^4	1.2×10^5	9.1×10^4	1.6×10^5	1.9×10^5
normalized[a]	1	0.98	1	0.83	1	0.76	1	1.19

[a] In each infection, the counts per minute at the two times are normalized to the earlier time.

sis of potyvirus-associated synergism is the ability of HC-Pro to prolong replication not only of potyviruses but also of a broad range of heterologous viruses.

Although our results suggest that synergism involves transactivation of viral replication, they do not rule out the possibility that the TEV sequence also facilitates viral movement. In fact, because the central domain of HC-Pro is required for both movement and replication of the potyvirus (Cronin et al., 1995; Kasschau et al., 1997) and the same domain is also required for the synergistic effect on PVX (Shi et al., 1997), a role in movement in addition to the role in replication remains quite possible.

Expression of the TEV P1/HC-Pro region affects TEV as well as a broad range of heterologous viruses. Therefore, we propose that synergism may occur via an indirect mechanism involving an interaction of the TEV-encoded proteins with one or more host factors common to the different viral infections rather than a direct mechanism involving interactions with different RNAs or proteins from three heterologous viruses.

Two different indirect mechanisms could explain transactivation of viral replication by P1/HC-Pro. The TEV sequence might increase the activity or availability of a positive regulator of viral replication that affects both TEV and heterologous viruses. The stimulation of one virus by a host factor induced by another virus has been shown in mixed infections with human cytomegalovirus and human immunodeficiency virus-1, in which human cytomegalovirus can induce expression of host transcription factor NF-κB, which then activates human immunodeficiency virus-1 replication (Chinnadurai, 1991; Kim et al., 1996).

Alternatively, the potyviral sequence might interfere with the activity or availability of a negative regulator of viral replication, perhaps part of a host defense system that normally limits viral accumulation. Because HC-Pro enhances the accumulation of a broad range of viruses and acts at the level of viral replication, the putative host defense system would necessarily be general in nature and act at the single-cell level. A host system consistent with these requirements has been proposed as the underlying mode of action in sense RNA–mediated virus resistance in transgenic plants (Lindbo et al., 1993; Smith et al., 1994; Mueller et al., 1995; Baulcombe, 1996). In this model, an RNA targeting system is activated by high-level expression of a transgene that contains viral sequences. Once activated, the system rapidly destroys the specific viral RNA target, whether the RNA is expressed from the transgene or from a viral RNA template during viral replication (Dougherty et al., 1994; Dougherty and Parks, 1995; Mueller et al., 1995; English et al., 1996; Goodwin et al., 1996). The same cellular system is also thought to be involved in post-transcriptional gene silencing of nonviral transgenes in plants (Ingelbrecht et al., 1994; de Carvalho Niebel et al., 1995; Baulcombe, 1996). It is possible that synergism impacts this RNA targeting system as well. In this case, the P1/HC-Pro sequence might interfere with the induction or the action of the pathway and thus allow a broad range of viruses to overstep the normal host-imposed limits of RNA accumulation.

METHODS

Viruses and Plants

The virus isolates used in these experiments are as follows: the common strain of tobacco mosaic virus (TMV) (ATCC PV-135) and cucumber mosaic virus (CMV-S), which was the kind gift of S. Scott (Clemson University, Clemson, SC). The U-6B and vector-only control plants are transgenic lines in *Nicotiana tabacum* cv Havana 425 and have been previously described (Carrington et al., 1990). Engineered potato virus X (PVX) viruses were prepared by infecting tobacco protoplasts with in vitro transcripts of the corresponding infectious cDNAs. Experiments using these engineered viruses were performed in *N. benthamiana*.

Inoculation and Sampling

Transgenic lines U-6B and vector-only control plants were mechanically inoculated with purified CMV or TMV at a concentration of 0.5 mg/mL. All plants were germinated and grown in an insect-free growth chamber, and plants for a given experiment were of the same size and developmental stage at the time of inoculation. Plants infected with TMV were incubated at 30 to 32°C to allow systemic spread of the virus, because *N. t.* Havana 425 carries the *N* gene, which mediates a hypersensitive response and localizes TMV at temperatures <28°C (Whitham et al., 1994). Total nucleic acid was isolated from equivalent systematically infected leaves of the U-6B line and vector-only control plants by using a guanidine–HCl procedure (Vance, 1991). Levels of viral RNAs were determined by RNA gel blot analysis, as described below.

Infectious Viral cDNAs

An infectious cDNA clone (pTXS; Kavanagh et al., 1992) of the UK-3 strain of PVX was the kind gift of D. Baulcombe (The Sainsbury Laboratory, Norwich, UK) and served as the starting point for construction of infectious PVX cDNAs carrying foreign inserts. To construct PVX-HC, the tobacco etch virus (TEV) HC-Pro gene was amplified from TEV cDNA clone pTL7SN.3-0027 (Verchot et al., 1991) by using polymerase chain reaction (PCR) with high-fidelity Vent polymerase (New England Biolabs, Beverly, MA). Two PCR primers were used: one was complementary to the 3′ proximal region of the HC-Pro coding region, but with an added translation stop codon (TGA) followed by a sequence specifying an XhoI restriction enzyme site. The other primer was complementary to the 5′ proximal portion of the proteolytically processed HC-Pro, but with 12 added upstream nucleotides, GAUAUCAUGGCC, encoding an EcoRV restriction site for cloning purposes and a translation start site followed by an alanine codon to provide a good context for translational initiation in plants (Lutcke et al., 1987).

Thus, translation of the sequence encoding HC-Pro on PVX-HC produces a protein with two extra N-terminal amino acids (methionine

and alanine) compared with authentic HC-Pro. The PCR fragment was cut with EcoRV and XhoI and ligated into EcoRV-XhoI–cut pTXS-luc, a derivative of pTXS (Sriskanda et al., 1996), to put the TEV HC-Pro gene under control of the PVX coat protein promoter in place of the coat protein gene. To put the coat protein gene back into the construct, a DNA fragment containing 40 nucleotides of coat protein subgenomic promoter sequence, the PVX coat protein coding sequence, the entire PVX 3′ untranslated region (UTR) plus poly(A) tail, and vector sequence up to the SacI site was amplified from pTXS by using the amplification primers to add a SalI site upstream of the PVX coat protein promoter and replace the SpeI site in the vector with an MluI site. This amplified SalI-SacI fragment was ligated into the previous construct at the XhoI and SacI sites to produce pTXS-HC.

The SpeI site, which is located at the end of the PVX sequence in pTXS and used to linearize that plasmid for in vitro transcription, was replaced with an MluI site in pTXS-HC, because the HC-Pro coding sequence contains two SpeI sites. Linearization of pTXS-HC with MluI and transcription from the T7 promoter result in an infectious RNA transcript with 5′ and 3′ termini identical to those of authentic PVX genomic RNA.

Thus, the PVX-HC cDNA clone has the original pTXS sequence up to the EcoRV site, followed by the TEV HC-Pro coding region with added translation start and stop sites, followed by the pTXS sequence comprising the region 40 nucleotides upstream of the coat protein AUG through the coat protein coding region and including the SacI site in the downstream polylinker of the vector. Because both HC-Pro and PVX coat protein are expressed from the coat protein subgenomic promoter, nucleotides 5620 to 5660 of pTXS are duplicated in the construct.

The plasmid pTXS-noHC was constructed in exactly the same manner as pPVX-HC, except that the PCR primer used to amplify TEV HC-Pro had ACG in place of the translation start site ATG to eliminate translation of the HC-Pro gene. pTXS-5′TEV was produced from pTXS-HC by replacing the HC-Pro gene and most of the coat protein gene with a PCR-amplified EcoRV-XhoI fragment containing nucleotides 146 to 2674 of the TEV genome. The PVX sequence specifying the coat protein promoter, coat protein coding sequence, and 3′ UTR was then replaced as described for pTXS-HC. The organization of the infectious viral RNAs derived by in vitro transcription of these cDNAs is shown in Figure 3A.

The PVX reporter virus PVX-5′TEVluc was constructed by using PCR to amplify the luciferase encoding region of pTXS-luc (Sriskanda et al., 1996) and then digesting the PCR-amplified fragment with SalI and XhoI and cloning it into the XhoI site of a precursor of pTXS-5′TEV lacking most of the PVX coat protein coding sequence. The K mutation was introduced by replacing an internal SpeI fragment within the HC-Pro coding region of pTXS-5′TEVluc with the corresponding fragment from a TEV cDNA containing a nine-nucleotide insertion that introduced an NcoI site and resulted in the insertion of the amino acid triplet Thr-Met-Ala immediately after amino acid 426 of the expressed TEV polyprotein (Shi et al., 1997).

Protoplast Infection System

Protoplasts from NT-1 tobacco suspension culture cells (An, 1985) were infected with in vitro transcripts of the infectious engineered PVX cDNAs. Capped transcripts were synthesized using the Ribomax T7 transcription kit (Promega) with the GTP concentration reduced to 3.75 mM and the addition of 3.75 mM CAP analog (New England Biolabs). Protoplasts were prepared from NT-1 cells at the logarithmic stage of growth and electroporated with viral transcripts exactly as previously described (Sriskanda et al., 1996). The levels of PVX (+) and (−) strand RNAs at various times after electroporation were determined by RNA gel blot analysis with strand-specific probes, as described below. Luciferase activity from protoplasts infected with either PVX-5′TEVluc or PVX-5′TEV(K)luc was assayed as previously described (Sriskanda et al., 1996) by using a luciferase assay kit (Promega). Luciferase activity was determined for cell lysates from ~30,000 protoplasts.

RNA and Protein Gel Blot Analyses

Total nucleic acids were isolated from leaves or protoplasts and analyzed by using RNA gel blots exactly as previously described (Vance, 1991). Radioactive probes complementary to the entire PVX or TMV (+) strand RNA were generated by using reverse transcriptase, hexanucleotide random primers, and either PVX or TMV genomic RNA isolated from purified virus. Probe specific to the (−) strand of PVX was generated by limited base hydrolysis (10 min at 65°C in Tris-HCl, pH 9.5) of purified PVX genomic RNA, followed by ethanol precipitation and 5′ end labeling with T4 polynucleotide kinase and γ-^{32}P-ATP (Maizels, 1976). Specificity of the PVX (−) strand probe was confirmed by its failure to hybridize to a sample containing microgram amounts of PVX genomic RNA included on the blot (Vance, 1991). Radioactive transcripts specific for CMV viral RNAs were generated by in vitro transcription of a CMV cDNA clone from the T7 promoter to produce a 200-nucleotide transcript complementary to the 3′ proximal region common to CMV viral RNAs 1, 2, 3, and 4 (Rizzo and Palukaitis, 1990). Radioactive transcripts complementary to the (+) strand of CMV satellite RNA were generated by in vitro transcription of a cDNA clone (ATCC 45125) from the T7 promoter. The levels of viral RNAs on RNA gel blots were quantitated by densitometric scanning of the x-ray film (at the appropriate exposure to be within the linear range of the film) by using a Microscan 1000 scanner (Technology Resources, Inc., Nashville, TN).

Levels of HC-Pro in systemically infected leaves were assayed by protein gel blot analysis. Total protein was extracted, quantitated, separated by SDS-PAGE (Vance, 1991), and transferred to polyvinylidene difluoride membrane (Bio-Rad) by using a Bio-Rad mini-trans-blot apparatus at 80 V for 1 hr. Rabbit polyclonal antiserum specific to TEV HC-Pro (Carrington et al., 1990) was used as primary antiserum, and immunoreactive proteins were visualized by using a goat anti–rabbit antiserum conjugated to alkaline phosphatase (Bio-Rad).

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